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Chemical and Immunological Characterization of a Low Molecular Weight Outer Membrane Protein of *Salmonella typhi*

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Abstract: **A new immunogenic outer membrane protein, Omp-28 (MW 28,000 and pI 4.6), was isolated from smooth *Salmonella typhi* cells by the use of an extracting medium containing 6 M urea, 1% deoxycholate and 5 mM EDTA. The purification of Omp-28 was performed by gel filtration and fast ion exchange chromatography. This protein showed to be the prevalent component isolated by the latter methodology. Omp-28 is formed by three identical subunits (MW 9,000), not linked by disulfide bonds. The partial N-terminal amino acid sequence of Omp-28 presented great homology with part of the sequence of an *Escherichia coli* protein found in a precursor whose sequence was predicted by c-DNA. ELISA and Western blotting identified Omp-28 as the major antigenic protein present in the outer membrane protein fraction, isolated by gel filtration. Antibodies against Omp-28 were detected by ELISA in 43% of 28 sera from typhoid fever convalescent patients. The antisera from mice immunized with Omp-28 and the highest positive typhoid fever convalescent serum gave a positive bactericidal test, killing 50% of *Salmonella typhi* cells in serum dilutions of 1/80 and 1/320, respectively. These results indicate the immunogenic importance of Omp-28 isolated from *Salmonella typhi* outer membrane and strongly suggest it should be used in further studies of animal protection against the disease caused by this pathogenic bacteria.**

Key words: ***Salmonella typhi*, Outer membrane protein, Purification, Characterization**

The attempts to find a vaccine from proteins rather than from polysaccharide against *Salmonella typhi* infection has showed that outer membrane proteins are promisor antigens (8, 9). In porins in recent years, a structurally well conserved and ubiquitous protein of Gram-negative bacteria outer membrane has been isolated and immunologically characterized, suggesting its use as a potential vaccinal immunogen (2, 3, 11, 12, 19). The porins (Omp A, Omp C, Omp F and others) are described as proteins of approximately 130,000 Da in molecular weight and formed by three identical subunits (17). These proteins span the bacteria outer membrane in its β-strand conformation, and the bulk of these structures is immersed in the lipopolysaccharide and peptidoglycan layers. Many of the porin preparations isolated from *Salmonella* sp. present slight contamination of lipopolysaccharide (LPS) and the true role of porins as powerfull antigens has been questioned (10, 11). Using *S. typhimurium*, investigations were performed to characterize the ability of porins and porin-LPS to confer effective protection in mice against *Salmonella* infection. The results demonstrated that the transfer of serum obtained from porin-LPS immunized mice resulted in better protection than did anti-LPS or anti-porin antibodies alone (11).

Immunoblotting after SDS-PAGE showed that other

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Abbreviations: DAB, diamine benzidine; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FPLC, fast-performance liquid chromatography; HBSS, Hanks buffered salt solution; HPLC, high-performance liquid chromatography; IEF-PAGE, Isoelectric focusing in polyacrylamide gel electrophoresis; OMP, outer membrane protein; PTH, phenylthiohydantoin; RP, reverse phase; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TMB, tetramethylbenzidine.

relevant proteins are also present in the outer membrane of *Salmonella* sp., as the 55,000 Da molecular weight subunit that evoked protective antibodies (6) and the 28,000 Da molecular weight subunit which reacted with sera from patients in the acute phase of typhoid fever (12).

In this paper, the isolation and characterization of a new immunogenic low molecular weight outer membrane protein from *S. typhi* is reported.

Materials and Methods

Bacteria and growth conditions. *Salmonella typhi* (strain S.2154, kindly gifted by Dr. Ernesto Hofer from IOC/FIOCRUZ, Rio de Janeiro, Brazil) was grown under anaerobic conditions according to Foulaki et al (6). Exponentially grown cells were harvested by centrifugation (4,000×*g* for 30 min at 5 C), washed with 10 mM Tricine buffer, pH 7.2, and centrifuged again. The pellet was immediatly used for outer membrane protein extraction.

Outer membrane protein (OMP) fraction isolation. Bacterial cells were suspended in a 10 mM Tris HCl buffer, pH 7.2, containing 6 M urea, 1% deoxycholate and 5 mM EDTA, and stirred at 42 C for 2 hr according to Foulaki et al (6) with slight modifications. After centrifugation (17,000×*g*, 30 min), the supernatant was separated and stored at −10 C. The supernatant was desalted in a Bio-gel P-6 column (1.5×30 cm), dried in a Speed Vac (Savant), and chromatographed in a Sepharose CL 6B column (2.5×60 cm), being eluted with 10 mM Tris HCl buffer, pH 7.5, containing 5 mM EDTA and 0.1 M NaCl (1). The OMP obtained was desalted as above.

Isolation of Omp-28 by FPLC-mono Q. The OMP preparation was fractionated by fast ion exchange chromatography in a mono Q column (1.0×10 cm) (Pharmacia Biotech). The elution used a NaCl linear gradient (0–0.5 M) performed in 100 mM Tris HCl buffer, pH 8.0. The main component, observed at 280 and 215 nm was concentrated, desalted and lyophylized. The fraction obtained was submitted to another mono Q ion exchange chromatography under the same conditions, desalted and dried in a Speed Vac. This fraction was named Omp-28. Further conditions are described in the legend of Fig. 1, panel A.

Homogeneity. The homogeneity of Omp-28 was ascertained by discontinuous native and denaturing polyacrylamide gel electrophoresis and reverse-phase chromatography. Discontinuous polyacrylamide gel electrophoresis-gradient system (8–25%) was performed in a Phast System (Pharmacia Biotech). The isoelectric focusing in the polyacrylamide gel (7%) and pH range (2.5–6.0) was performed in a Multiphor II System (Pharmacia Biotech). The staining and destaining were carried out as recommended by manufacturers. Tricine sodium dodecyl sulfate-polyacrylamide gel (16.5%) electrophoresis, according to Shägger and Jagow (15), was performed in a miniProtean II (Bio-Rad Lab). HPLC-RP was performed in a C-4 column (1.0×25 cm, Bio-Rad Lab); for more details, see Fig. 1, panel C.

Isoelectric point and molecular weight estimations. The isoelectric point was determined by isoelectric focusing as previously described and FPLC-chromatofocusing in a mono P column (HR 5/20, Pharmacia Biotech) using a decreasing pH gradient formed by the addition of polybuffer 74 HCl solution (1/15 v/v, pH 3.85) to the 25 mM Imidazol HCl buffer solution pH 5.50. The flow rate was 0.7 ml/min and detection was performed at 280 nm. Omp-28 had its molecular weight estimated by a FPLC-Superdex 75 (1.2×60 cm, Pharmacia Biotech) and a HPLC-Protein-pak 300 SW (0.78×30 cm, Waters Co.). In the first chromatography, the protein (2 mg) was eluted with 100 mM Tris HCl buffer, pH 7.5, containing 10 mM EDTA and 0.15 M NaCl. In the second chromatography, the protein (1 mg) was eluted with 50 mM phosphate buffer, pH 6.0, containing 0.15 M NaCl. In both experiments, the flow rate was 0.5 ml/min and all detections were performed at 215 and 280 nm in a Photo Dyode Array (Waters Co.). The molecular weights of the polypeptide chains or subunits present in Omp-28 were determined by Tricine-SDS-PAGE 16.5% (15).

Protein and carbohydrate quantification. A modified Folin-Lowry method (13) was employed for protein quantification, using bovine serum albumin as the standard. The classical phenol-sulfuric method (5) was used for the determination of neutral carbohydrates present in the isolated protein.

Amino acid analysis, sequencing and homology. The protein Omp-28 was submitted to amino acid analysis in a Biochrom 20 amino acid analyzer (Pharmacia Biotech). The protein (1 mg per hydrolyzate) was submitted to acid hydrolysis (HCl 5.7 N, 110 C, 24, 45 and 72 hr). Two analyses of the protein were carried out in each hydrolyzate. Tryptophan was not quantified.

The amino acid sequencing of Omp-28 was performed in an automatic gas-liquid sequenator (Shimadzu). Fifty microlitres of Omp-28 (0.56 mg per ml) were immobilized in the polybrene membrane of the sequenator and submitted to several degradative cycles. For comparing the N-terminal partial amino acid sequence of Omp-28 and other homologous proteins, the SWISS-PROT databank was used.

Immunization schedule. Two groups of ten mice weighing 15–17 g each were immunized with 5 µg of

OMP for one group and 5 μg of Omp-28 for the other. Both antigens were mixed in incomplete Freund's adjuvant and injected subcutaneously on days 0 and 14. Seven days after the second immunization, all mice were boosted intraperitonealy (i.p) with the same protein fractions (5 μg of each) diluted in phosphate-buffered saline (PBS). Three days later, the mice were bled, and the sera collected, pooled and stored at −20 C until use.

ELISA. ELISA plates were coated with OMP or Omp-28 and assayed with serum antibodies of the IgG class using horseradish peroxidase-conjugated anti-mouse and anti-human IgG immunoglobulins. After color development with H_2O_2 (0.006%) and TMB (0.1%), the reaction was stopped and the plates were read at 450 nm in an ELISA reader (Titertek Multiscan Plus).

Western blot analysis. OMP and Omp-28 were electroblotted from Tricine-SDS-PAGE 16.5% onto nitrocellulose membrane by the method of Towbin et al (18). The immune reaction was performed utilizing, as first antibodies, sera obtained from immunized mice (see above) and sera from typhoid fever convalescents. As second antibodies, anti-mouse IgG (1/3,000, v/v) and anti-human IgG (1/1,500, v/v) conjugated to peroxidase (Sigma Co.) diluted in phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20 were used. Incubation was performed at 37 C for 2 hr. Finally, the development used H_2O_2 (0.006%) and DAB (0.25 mg/ml) in 150 mM PBS, pH 7.2, for 5 min. Densitometric analyses of gels and nitrocellulose strips were performed in an Image Master System (Pharmacia Biotech).

In vitro *bactericidal activity.* The test was made according to Saxena, M. and Di Fabio, J. (14). Briefly, *S. typhi* was grown in brain-heart infusion (BHI), pelleted by centrifugation, washed twice with physiological saline and adjusted to 2×10^3 colony-forming units per ml in Hanks buffered salt solution (HBSS). Different dilutions of the test sera (25 μl) were mixed with a bacterial suspension (25 μl) and guinea-pig complement (25 μl). The reaction mixture was incubated at 37 C, and after 1 hr, the suspension was plated onto Müller Hinton Agar and incubated at 37 C overnight. The bactericidal titers were calculated as the maximum dilution of serum required to kill 50% of the initial inoculum. Non-specific killing due to guinea-pig complement was controlled by inoculation of 25 μl of cell suspension with 25 μl of HBSS plus 25 μl of guinea-pig complement plated onto the Müller Hinton Agar.

Results

An outer membrane protein fraction prepared according to Bhatnagar et al (1) was used for the isolation of Omp-28 by FPLC-mono Q (Fig. 1, panel A). The protein preparation so obtained showed slight heterogeneity when analyzed by native and denaturing polyacrylamide gel electrophoreses, and was then submitted to repurification using the same anionic support. The repurified protein Omp-28 (yield 38%) was considered homogeneous by Tricine-SDS-PAGE 16.5% (Fig. 1, panel B) and reverse-phase C4-chromatography (Fig. 1, panel C) as well as by isoelectric focusing (pH range 4.0–6.5), discontinuous-PAGE 12.5%, FPLC-mono P chromatofocusing (pH range 5.50–3.85) and Western blotting (Fig. 1, panel D). Its isoelectric point calculated by IEF-PAGE was 4.6, and by FPLC-chromatofocusing 4.3. Its molecular weights calculated by FPLC-Superdex 75 and HPLC-Protein-pak 300 SW were 27,000 and 29,000 Da, respectively; whereas the value of the molecular weight of its identical subunits, not linked by disulfide bridges, was estimated to be 9,000 Da by Tricine-SDS-PAGE 16.5% (Fig. 1, panel B). An amino acid analysis of Omp-28 showed the following minimal composition: D_{10}, T_7, S_4, E_{13}, P_4, G_4, A_7, C_2, V_7, M_2, I_3, L_6, Y_1, F_3, K_8, R_2. This protein lacks histidine, and tryptophan was not determined. The protein recovery was superior to 94% according to this analysis. Omp-28 seems to be devoid of carbohydrates as demonstrated by the phenol-sulfuric acid method (<1%). Partial amino acid N-terminal sequencing of the Omp-28 subunit performed by a Shimadzu sequenator gave A^1-T-D-T-T-K-T-N-V-T-P-K^{12}-G-M-S-?-Q-E-F-V-D-L-N-P^{24}. The initial yield observed for alanine was 58% assuming 9,000 Da as the Omp-28 subunit molecular weight (Fig. 1, panels B and D). Therefore, the Omp-28 protein has only one type of polypeptide chain. The amino acid sequence of the polypeptide chain of Omp-28 presented great homology with a protein of *E. coli* (protein HDEB precursor-10 K-L protein), mainly from the 12th up to 24th residue, where the sequences are practically the same (16, 20, 21). Furthermore, the *E. coli* protein sequence in the homologous region (K^{39}-D-M-T-C-Q-E-F-I-D-L-N-P^{51}) suggested that the space observed in the Omp-28 sequence must be occupied by a cysteine residue since the low value of serine observed in the 16th sequential step must be related to PTH-Cys destruction.

Antibody levels in the sera from mice immunized with *S. typhi* OMP and Omp-28 were analyzed by ELISA using OMP as the antigen. The titer observed for both sera was 1:10,240. These sera were also analyzed by immunoblotting (Fig. 1, panel D), and the results observed show the relevance of the Omp-28 as the major antigen present in the *S. typhi* cell envelope, since only the 9 kDa subunit was recognized by both anti-sera: anti-Omp-28 mouse immune serum and anti-OMP mouse immune serum.

Antibody levels in sera from humans infected with *S.*

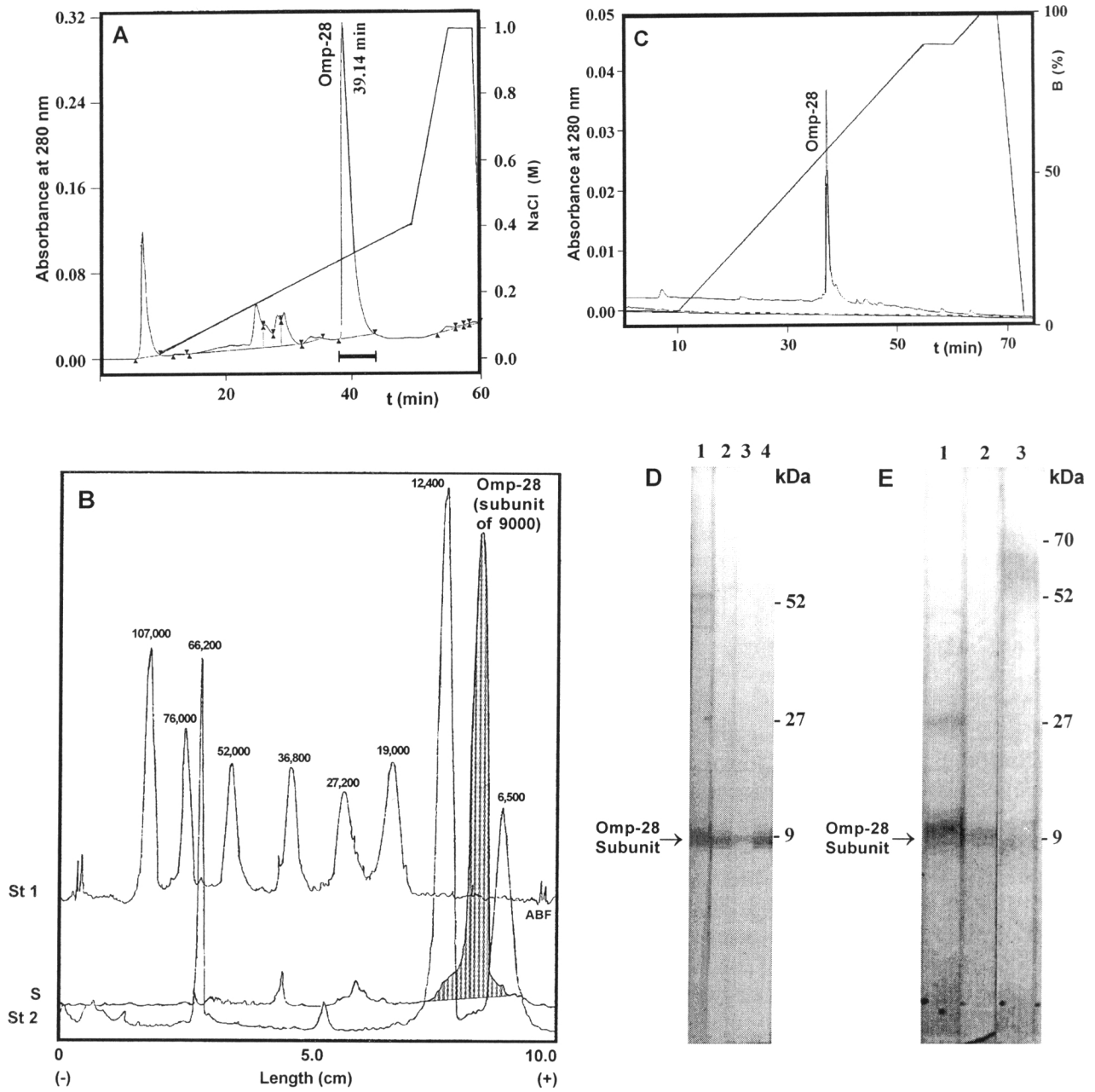

Fig. 1. Isolation (panel A), homogeneity (panels B and C) and immunological characterization (panels D and E) of Omp-28. A: FPLC-mono Q of the outer membrane protein (OMP) fraction. A sample (2 mg) was dissolved in 5 ml of 100 mM Tris HCl equilibrium buffer, pH 8.0, and chromatographed for 10 min with this solution followed by a linear NaCl gradient (0–0.5 M) for 40 min and (0.5–1.0 M) for 3 min. This gradient was performed by the addition of 100 mM Tris HCl buffer, pH 8.0, containing 1.0 M NaCl to the equilibrium buffer. Finally, after 3 min of elution with the terminating buffer, the column was re-equilibrated with the starting buffer. No peak was observed after 60 min of elution. The flow rate was 0.7 ml/min and the fraction eluted at 39.14 min was collected. B: Densitometric analysis of Omp-28, 2 sets of standard proteins (bovine serum albumin, cytochrome *c* and aprotinin not pre-stained) and low molecular weight range pre-stained markers from Bio-Rad (lines St 1 and St 2, respectively). The molecular weight of the Omp-28 subunit observed after SDS or SDS+DTT incubation was estimated as 9,000 Da (line S). C: HPLC-RP-C4 of Omp-28 prepared from rechromatography in a mono Q column. A sample (1 mg) was dissolved in 0.5 ml of 0.1% TFA equilibrium solution and eluted for 10 min with this solvent followed by a linear acetonitrile gradient (0–90%B) for 45 min. The elution continued for 5 min with 90%B, and 5 (90–100%B) and 3 min with 100%B (B=90% acetonitrile in 0.08% TFA). After 5 min, the column was re-equilibrated under the initial conditions. The flow rate was 2 ml/min, and detection was performed at 215 and 280 nm. D: Western blotting of OMP fraction (lanes 1 and 2) and Omp-28 (lanes 3 and 4). After Tricine-SDS-PAGE 16.5%, the electroblotted samples were immunoreacted with OMP mouse antiserum (lanes 1 and 3) and Omp-28 mouse antiserum (lanes 2 and 4). E: Western blotting of OMP fraction (lanes 1 and 3) and Omp-28 (lane 2). After Tricine-SDS-PAGE 16.5%, the electroblotted samples were immunoreacted with typhoid fever convalescent patient sera (serum 163, lanes 1 and 2; serum 165, lane 3).

Table 1. ELISA of Omp-28 and OMP fractions

	Serum dilution						
(A) Omp-28	Blank	1/40	1/80	1/160	1/320	1/640	1/1,280
Normal serum	0.097	0.185	0.122	0.075			
Serum 3	0.096	0.259	0.224	0.123			
Serum 4	0.093	0.373	0.316	0.216	0.120		
Serum 5	0.100	0.441	0.347	0.306	0.207	0.125	
Serum 7	0.093	0.312	0.224	0.134	0.091	0.069	
Serum 9	0.095	0.284	0.102				
Serum 163	0.093	1.005	0.949	0.933	0.764	0.570	0.453
Serum 165	0.096	0.304	0.185	0.128			
(B) OMP							
Normal serum	0.094	0.075	0.052				
Serum 4	0.106	0.104					
Serum 5	0.103	0.109					
Serum 9	0.100	0.113					
Serum 163	0.093	0.440	0.357	0.293	0.310	0.184	0.114
Serum 165	0.093	0.135					

Normal serum and seven different typhoid fever convalescent patients sera obtained from the Institute Adolfo Lutz SP, Br. (a gift of Dr. Kinue Irino) were used. One microgram of each protein fraction was fixed in the plate wells. Sera Nos. 3 and 7 only recognized the Omp-28 protein.

typhi in the convalescent phase were analyzed by ELISA using Omp-28 and OMP as the antigen. Twelve of these sera (in a total of 28) recognized Omp-28 as the antigen. Table 1 depicts the values obtained by ELISA for only seven of these positive sera which recognized Omp-28 as the antigen more strongly, and a comparison of this data with that obtained when OMP was used as the antigen. For the same sera dilution, a higher value was always obtained for Omp-28. Convalescent serum No. 163 gave the highest value observed. In order to determine the antigenicity of Omp-28 in humans, antibodies against this protein were detected by immunoblotting in sera of patients with typhoid fever in the convalescent phase (Fig. 1E). The antibodies present in the human serum (No. 163) were directed mainly to the Omp-28 subunit (lanes 1 and 2), and at a lower level to the porin subunits (37–41 kDa) and two other bands (28 and 55 kDa) (lane 1). Another human serum (No. 165) detected the Omp-28 subunit band with the same intensity as serum 163 and another diffuse band between 52 and 70 kDa (lane 3).

The bactericidal test for *S. typhi* showed a titer of 1/80 for Omp-28 mouse antiserum and a titer of 1/320 for the highest positive convalescent serum.

Discussion

This paper describes the purification and characterization of a 28 kDa protein, Omp-28, isolated from *S. typhi* outer membrane. This protein was extracted from the bacteria by the use of a buffered solution containing 6 M urea, 5 mM EDTA and 1% deoxycholate. The association of a protein pertubant (urea) with a chelating agent (EDTA) and a detergent (bile salt) appears to provide the most generally useful extraction method presently available for the solubilization of proteins which are tightly bound to the lipid matrix of the cellular envelope (7). Gel filtration on Sepharose CL 6 B was used in order to obtain the outer membrane protein (OMP) fraction free of lipopolysaccharide (LPS) and nucleic acids (1). The homogeneity of the Omp-28 protein obtained by FPLC-mono Q rechromatography was ascertained by several purity criteria.

Among the *S. typhi* outer membrane proteins, porins are the best studied, and demonstrated to have some immunogenicity (2, 12). They have been isolated from bacteria envelopes by Triton/EDTA buffered solution, pH 7.4, and analyzed by Laemmli's SDS-PAGE (12, 17, 19). Like porins, the Omp-28 protein was purified as a primer. The molecular weight of its subunit is 9,000 Da according to Tricine-SDS-PAGE 16.5%, whereas the present values of molecular weights for porin (Omp C, Omp F, Omp D and others) subunits are in the range of 36,000–42,000 Da as determined by SDS-PAGE (17). The use of the classic Laemmli's SDS-PAGE in order to analyze the proteins present in the outer membrane of Gram-negative bacteria does not permit the resolution of its polypeptide chains with low molecular weights ($<$12,000 Da) (15). The isoelectric point of Omp-28 was determined to be 4.6, similar to the isoelectric point described for porins (10). Probably, Omp-28 is devoid of carbohydrates as demonstrated by Dubois' methodology

together with the high recuperation of this protein by amino acid analysis. The amino acid sequence of the 24 residues of Omp-28 is quite different from that presented by the porins (4). Furthermore, porins do not present cysteinyl residues in its structure, unlike the Omp-28 protein which presents about 2 residues of this amino acid per subunit. Part of one protein (10 K-L protein) present in the *E. coli* precursor showed great sequential homology with part (12th to the 24th amino acid residues) of the N-terminal amino acid sequence of Omp-28.

Western blotting and ELISA analysis of Omp-28 showed that this protein is the main immunogenic component present in the outer membrane protein fraction isolated from *S. typhi*. By ELISA, 43% of the sera of convalescent typhoid fever patients tested (in a total of 28) recognized the Omp-28 protein as the major antigen. Coincidently, all the other sera (57% of the total) which did not recognize Omp-28 as an antigen, gave high titers for the polysaccharide antigens O and H in a Widal test (results not showed), suggesting that such patients could still be considered in the acute phase of typhoid fever disease. The capacity of the sera of the mice immunized with Omp-28 to kill a colony of *S. typhi* was demonstrated and compared to the same capacity of the highest positive convalescent serum of typhoid fever patient (serum No. 163).

The data presented in this paper show that Omp-28 from the *S. typhi* envelope is an important immunogenic protein, and is suitable to be used in further protective experimental tests.

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